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(54) Title: MONOCLONAL ANTIBODY AND IMMUNOCONJUGATES FOR THE TREATMENT AND DETECTION OF B CELL DISORDERS

(57) Abstract

Methods and compositions for the treatment of B cell disorders are disclosed. B cell disorders may be treated through the use of an antibody that selectively binds to the surface immunoglobulin (sIg) of a target B cell, in combination with an immunoconjugate comprising at least one therapeutic agent coupled to an antibody that selectively binds to the CD19 antigen on B cells. The present invention also discloses methods and compositions for the detection of B cell disorders.

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Description

Monoclonal antibody and immunoconjugates for the treatment and detection of B cell disorders.

Technical Field

The present invention relates generally to compositions for use within methods for the treatment and detection of B cell disorders and, more specifically, to the use of immunoconjugates, formed from at least one agent and an anti-CD19 antibody, in combination with an antibody specific for the surface immunoglobulin of a target B cell.

Background of the Invention

Despite enormous investments of financial and human resources, cancer remains one of the major causes of death. One type of cancer is malignancies of B cells, e.g., Hodgkin's lymphoma, Burkitt's lymphoma and chronic lymphocytic leukemia. These malignancies represent 5% of all cancer deaths in the United States. Conventional forms of therapy, such as radiation and chemotherapy, have had limited success.

In addition to malignancies of B cells, other B cell disorders have posed problems in treatment. Autoimmune diseases are examples of such non-cancer B cell disorders. Examples of autoimmune diseases include rheumatoid arthritis, Graves' disease and myasthenia gravis.

Due to the difficulties in the current approaches to the treatment of B cell disorders, there is a need in the art for improved methods and compositions. The present invention fills this need, and further provides other related advantages.

Summary of the Invention

Briefly stated, in one aspect, the present invention provides an immunoconjugate for use within a method for treating a B cell disorder in a warm-blooded animal. The immunoconjugates comprise at least one therapeutic agent coupled to an antibody that selectively binds to the CD19 antigen, in combination with an antibody that selectively binds to the surface immunoglobulin of a target B cell.

In another aspect, the present invention provides an immunoconjugate for use within a method for detecting a B cell associated with a

B cell disorder. The immunoconjugates comprise at least one diagnostic agent coupled to an antibody that selectively binds to the CD19 antigen, in combination with an antibody that selectively binds to the surface immunoglobulin of a target B cell.

Within a related aspect of the present invention, a representative monoclonal antibody (MAb), J3-119, that selectively binds to the CD19 antigen is provided. A cell line, designated by ATCC No. HB 10383, that produces J3-119 is also provided.

In another related aspect, the present invention provides an immunoconjugate comprising at least one agent coupled to the monoclonal antibody J3-119 or a monoclonal antibody that competitively inhibits the formation of an immunocomplex between J3-119 and the CD19 antigen. Preferred agents include therapeutic and diagnostic agents.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 graphically illustrates that anti-CD19 MAb is internalized and degraded during anti-Ig-induced comodulation of CD19. Identical cultures of SKLY18 B lymphoma cells are incubated in parallel with ^{125}I-labeled anti-CD19 MAb (J3-119). Cells are also incubated with 15 μgm/ml of anti-CD4 MAb (Figure 1A) or anti-Ig MAb (Figure 1B). All other conditions are the same. Aliquots from the two cultures are removed at the times indicated and assayed for radiolabeled internalized, cell-bound, and free MAb (intact and antibody fragments).

Figure 2 graphically illustrates that anti-Ig MAb is rapidly internalized and degraded when cells are incubated with anti-Ig MAb at 37°C. SKLY18 B lymphoma cells are incubated with ^{125}I-labeled anti-Ig MAb alone at 37°C as described in Figure 1. Aliquots are removed at the times indicated and assayed for internalized, cell-bound, and free MAb (intact and antibody fragments).

Figure 3 pictorially depicts cocapping studies which confirm that anti-CD19 MAb is internalized and degraded when cells are incubated with anti-Ig MAb. Cells are pretreated with fluoresceinated anti-CD19 MAb at 4°C, washed, and coincubated with either isotype-matched anti-CD4 MAb (Panels A-D) or anti-lambda MAb (Panels E-H) at 37°C. Cells were removed, washed, and

analyzed at 0, 20, 40, 60, 90, and 120 minutes. Results at 40, 60, 90, and 120 minutes are indicated in panels A and E, B and F, C and G, and D and H, respectively.

Figure 4 graphically illustrates that coincubation of cells with both anti-Ig and anti-CD19 MAb selectively increases the rate and extent of CD19 modulation. CD19 expression is monitored on SKLY18 cells incubated with the following MAb (each at 10 μgm/ml) at 37°C: anti-CD4 (J5-19), anti-Ig (HB43), anti-CD19 (J3-119), and anti-Ig plus anti-CD19. The percent of detectable CD19 remaining on the cell surface at 2.25 and 5 hours is indicated.

Detailed Description of the Invention

As noted above, the present invention is directed toward methods and compositions for the treatment and detection of B cell disorders. The disclosure of the present invention shows that the combination of an antibody to surface immunoglobulins on B cells and an antibody to the CD19 antigen on B cells can be used for the rapid and specific delivery of agents, via CD19-specific immunoconjugates, to the intracellular compartment of targeted B cells.

B lymphocytes ("B cells") are responsible for the ultimate production of antibody molecules. B cells express both surface immunoglobulin (sIg) and non-immunoglobulin cell surface markers. sIgs recognize and bind antigens. The structure of sIg, like any immunoglobulin molecule, is composed of two heavy polypeptide chains and two light polypeptide chains. The heavy chains may be a gamma or mu type and the light chains a kappa or lambda type. In a given sIg molecule, all the heavy chains are identical and similarly all the light chains are identical. Light and heavy chains have both constant and variable regions, i.e., regions where the amino acid sequence is relatively the same from molecule to molecule, or relatively different, respectively. The variable regions of an immunoglobulin contain the structures responsible for the antigenic specificity of the immunoglobulins. The antigen binding site of every immunoglobulin molecule is structurally unique and, therefore, each immunoglobulin has unique antigenic determinants not shared by other immunoglobulin molecules. These unique determinants are one type of antigenic determinant ("idiotope") that distinguish a particular immunoglobulin. Other idiotopes may also be in the variable regions but outside the antigen-binding site. The collection of idiotopes (i.e., idiotype) distinguish immunoglobulins, and thus, B cells.

The CD19 antigen ("CD19") is a non-immunoglobulin 85-95 kilodalton cell surface protein residing on B cells. As disclosed within the present

invention, CD19 is a phosphorylated glycoprotein. CD19 is B cell-specific and has the broadest B cell reactivity of all known B cell surface proteins. It is common to all B cells except plasma cells (i.e., a pan-B surface antigen). A number of antibodies specific for CD19 ("anti-CD19 antibodies") have been reported.

As disclosed within the present invention, the combination of an antibody which binds to sIg on B cells and an antibody to CD19 (in the form of a CD19-specific immunoconjugate) can be used to achieve rapid and specific delivery of CD19-specific immunoconjugates to the intracellular compartment of targeted B cells. An advantage of this dual antibody approach is that the cytoplasm of a target cell is exposed to higher levels of CD19-specific immunoconjugates, and therefore of the transported diagnostic or therapeutic agent, than when the CD19-specific immunoconjugates are used alone. Moreover, only that subset of B cells which possess the chosen surface immunoglobulin molecules are exposed to this higher intracellular concentration of CD19-specific immunoconjugate, thereby giving the method a higher specificity than when the CD19-specific immunoconjugate is used alone. Another advantage is the accelerated uptake of CD19-specific immunoconjugates by target cells treated with both antibodies. This accelerated uptake may minimize toxicity of these immunoconjugates to scavenger cells of the reticuloendothelial system. Thus, the more rapid, specific, and efficient delivery of CD19-specific immunoconjugates to target cells by the dual antibody approach of the present invention increases the specificity and efficacy of these immunoconjugates while reducing their toxicity.

Within one aspect of the present invention, a B cell disorder may be treated by administering to a warm-blooded animal, such as a human, an effective amount of an antibody that selectively binds to the surface immunoglobulin of a target B cell, in combination with an immunoconjugate comprising at least one therapeutic agent coupled to an antibody that selectively binds to the CD19 antigen. This method is applicable to a wide variety of B cell disorders, including malignancies and autoimmune diseases. Representative B cell malignancies include non-Hodgkin's lymphoma, Burkitt's lymphoma, and chronic lymphocytic leukemia. Representative autoimmune diseases, i.e., diseases in which an individual is producing antibodies against one or more of its own proteins, include juvenile and adult rheumatoid arthritis, Graves' disease, and myasthenia gravis.

A variety of antibodies that selectively bind, i.e., with an affinity of about 10^7 liters/mol or higher, to sIg of a target B cell are suitable for use within this method of the present invention. In one embodiment, the antibody may be an anti-Ig heavy chain antibody, i.e., anti-gamma or anti-mu, or an anti-Ig light chain

antibody, i.e., anti-kappa or anti-lambda. The anti-Ig light or heavy chain antibodies may be polyclonal or monoclonal antibodies. Briefly, polyclonal antibodies may be produced by immunization of an animal and subsequent collection of its sera. It is generally preferred to follow the initial immunization with one or more boosters prior to sera collection.

While polyclonal antibodies which selectively bind to a particular Ig chain may be employed in the present invention, monoclonal antibodies (MAbs) are preferred. Suitable MAbs include those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (i.e., antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies may be produced using methods known by those skilled in the art. Human antibodies and chimeric human-mouse antibodies are advantageous because they are less likely than murine antibodies to cause the production of anti-antibodies when administered clinically.

MAbs may be generally produced by the method of Kohler and Milstein (Nature 256:495-497, 1975; Eur. J. Immunol. 6:511-519, 1976). Briefly, the lymph nodes and/or spleens of an animal immunized with an Ig chain are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin specific for the Ig chain, and, like the myeloma cells, has the potential for indefinite cell division. MAbs which selectively bind to a particular Ig chain are commercially available, e.g., MAb HB43 (1410KG7) specific for Ig gamma chain may be obtained from the American Type Culture Collection.

The particular anti-heavy chain antibody (i.e., anti-gamma versus anti-mu) or the particularly anti-light chain antibody (i.e., anti-kappa versus anti-lambda) selected depends upon the Ig phenotype, i.e., heavy and light chain specificity, of the target B cell. For example, since the cells of a B cell tumor are monoclonal in nature, all the cells of the target tumor will have the same Ig phenotype. Following routine isolation of malignant B cells (e.g., from sera, lymphatic fluid or a tumor mass), the Ig phenotype may be determined using one of a variety of methods known by those skilled in the art. For example, washed cells are incubated separately with a series of antibodies specific for immunoglobulin gamma, mu, kappa, or lambda chains or with nonreactive isotype-matched control antibodies or with antibodies specific for other B cell-associated antigens, for 30 minutes at 4°C. Cells are washed, and bound antibodies are detected, e.g., using a standard indirect immunofluorescence

binding assay. The immunoglobulin phenotype of the tumor cells is indicated by the specificity of the anti-immunoglobulin antibodies which they bind. When the cell sample consists of both normal and tumor cells, tumor cells are identified both by size and by their expression of tumor-associated antigens. Since tumor cells are monoclonal, they are further distinguished from normal B cells by their expression of a single immunoglobulin surface phenotype.

In another embodiment of the methods of the present invention for treating a B cell disorder, the antibody that selectively binds to sIg of a target B cell may be an anti-idiotype antibody. Following routine isolation of a sample of the B cells to be targeted for treatment, the intact cells or sIg isolated from the cells can be used to produce anti-idiotype antibodies. Briefly, Balb/c mice are immunized with either the purified idiotype antibody secreted by a patient's tumor or with a patient's tumor cells, depending on availability. Resulting hybridomas which secrete anti-idiotype antibodies are identified first by screening supernatants from hybridoma cultures for positive reactivity with the immunogen (idiotype antibody or tumor cell) using any one of a series of binding assays (e.g., immunofluorescence, radioimmune, or ELISA). Antibodies from reactive clones are further screened for negative reactivity with other purified human immunoglobulins, e.g., by radioimmunoassay. Antibodies which react with other purified human immunoglobulins are discarded. Those hybridomas which produce supernatants with antibodies which only react with the immunogen are further tested for binding to patient tumor cells which have been incubated with anti-immunoglobulin antibodies (i.e., anti-gamma, anti-mu, anti-lambda, anti-kappa) which remove or modulate the surface immunoglobulin on these cells. Anti-idiotype antibodies show decreased binding to cells whose surface immunoglobulin has been modulated. The antigen identified by the putative anti-idiotype antibody is determined (e.g., by immune precipitation) and compared with that identified by anti-immunoglobulin antibody on the same cells. Tumor cells from the patient are surface radiolabeled, e.g., with iodine (^{125}I), and solubilized in nonionic detergent. Surface immunoglobulin is precipitated by anti-idiotype and anti-immunoglobulin antibodies and analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Similarly, the target antigen of anti-idiotype antibodies is tested for cocapping by anti-immunoglobulin antibodies using standard procedures.

As noted above, the other antibody in the dual antibody methods of the present invention is an antibody, in the form of an immunoconjugate, that selectively binds, i.e., with an affinity of about 10^7 liters/mol or higher, to CD19.

Suitable anti-CD19 antibodies include antibodies provided by the present invention as well as those described by others. The anti-CD19 antibodies may be polyclonal or monoclonal. The discussion above regarding polyclonal and monoclonal antibodies applies equally here. The present invention provides a representative example of a suitable monoclonal antibody, J3-119, and a cell line, designated by ATCC No. HB 10383, that produces it. MAb J3-119 selectively binds to CD19 from a variety of organisms. The J3-119 anti-CD19 MAb is unique among CD19-specific antibodies in its ability to bind to the B cells of nonhuman primates (*M. Mulatta, M. Nemestrina, P. Cynocephalus*), just as it does in human.

An antibody that selectively binds to CD19 is coupled to one or more therapeutic agents to form a CD19-specific immunoconjugate. Suitable agents include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include ^{90}Y, ^{123}I, ^{125}I, ^{131}I, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi. Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diptheria toxin, cholera toxin, gelonin, *Pseudomonas* exotoxin, *Shigella* toxin, and pokeweed antiviral protein.

An agent may be coupled to, e.g., covalently bonded to, an anti-CD19 antibody either directly or indirectly, e.g., via a linker group. A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group, e.g., a halide, on the other.

Alternatively, it may be desirable to couple an agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also facilitate the use of agents, or functional groups on agents, which otherwise would not be possible. A carboxyl group, for example, may be activated. Activation of a carboxyl group includes formation of an "active ester," such as a succinimidyl ester. The term "active ester" is known to refer to esters which are highly reactive in nucleophilic substitution reactions.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be

employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, e.g., U.S. Patent No. 4,671,958, to Rodwell et al. (this patent and those disclosed below are herein incorporated by reference).

Where an agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described previously. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (e.g., U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (e.g., U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (e.g., U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (e.g., U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (e.g., U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an anti-CD19 antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers which provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (e.g., U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (e.g., U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (e.g., U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792, to Srivastava, and European Patent Application Publ. No. 0203764 (published December 3, 1986) disclose representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example,

U.S. Patent No. 4,673,562, to Davison et al., and European Patent Application Publ. No. 0188256 (published July 23, 1986) disclose representative chelating compounds and their synthesis.

An immunoconjugate such as those described above is administered in combination with an antibody that selectively binds to the sIg of a target B cell. A variety of routes of administration may be used. Typically, administration will be intravenous or intracavitary, e.g., in pleural or peritoneal cavities, or in the bed of a resected tumor. While simultaneous administration of an anti-sIg antibody and a CD19-specific immunoconjugate may be preferred for convenience, their administration may be sequential. The precise optimum dose for a particular anti-sIg antibody may vary, depending in part on the antibodies used, the antigen density on the tumor, the amount of cross-reactive circulating immunoglobulin, and the bulk of tumor present. The affinity and rate of clearance of individual antibodies from the body may also vary. Since anti-idiotype antibodies are more specific than anti-heavy or anti-light chain antibodies, the dosage may be less. Generally, however, an effective amount of an anti-sIg antibody will be from about 0.01 to about 100 mg per kg body weight. Similarly, the precise optimum dose for a particular CD19-specific immunoconjugate may vary. As above, the optimum immunoconjugate dose will vary with the antigen density on the tumor and the bulk of tumor present. Moreover, agents vary with respect to their potency and antibodies vary with respect to binding affinity. Generally, however, an effective amount of an immunoconjugate of the present invention will be from about 0.01 to about 10 mg per kg body weight. It will be evident to those skilled in the art how to determine the optimal effective doses for a particular anti-sIg antibody and for a particular CD19-specific immunoconjugate, and that the number and frequency of administration will be dependent upon the response of the patient. Suitable carriers or diluents include physiological saline.

Within another aspect of the present invention, a B cell disorder may be detected by administering to a warm-blooded animal, such as a human, an effective amount of an antibody that selectively binds to the surface immunoglobulin of a target B cell, in combination with an immunoconjugate comprising at least one diagnostic agent coupled to an antibody that selectively binds to the CD19 antigen. Typically, this method will be used to determine the location(s) of a B cell disorder or to monitor a patient for residual or recurrent targeted B cells. With the exception of the description of preferred therapeutic agents, the entire discussion above applies equally here. Suitable diagnostic agents include those which are detectable directly or indirectly. For example, for

direct detection, suitable diagnostic agents include a reporter group such as a radionuclide or magnetic imaging enhancer. Preferred radionuclides include ^{99m}Tc, ^{111}In, and ^{125}I. Preparation of a magnetic imaging enhancer coupled to an anti-CD19 antibody may be effected by a variety of methods (e.g., U.S. Patent No. 4,735,210, to Goldenberg). It will be evident to those skilled in the art that the method of detecting a diagnostic agent will be dependent upon the type of agent utilized. A variety of equipment, such as ionizing radiation detectors, is commercially available for the detection of diagnostic agents.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

Antibody Preparation and Conjugation

A. Cell Fusion and Screening

MAb HB43 (1410KG7) specific for immunoglobulin gamma chain was obtained from the American Type Culture Collection (ATCC, Rockville, Md.).

MAb J3-119 was prepared as follows. BALB/c mice were immunized with cells from the malignant human B cell line BALM-4, which was established from a patient with diffuse poorly differentiated lymphocytic lymphoma. These cells express sIgG-kappa, CD19, and CD20, confirming that they are of B cell origin. They are negative for the T cell-associated CD3, CD4, and CD8 antigens.

MAb J3-119 was produced by fusing spleen cells of immunized BALB/c mice with the murine plasmacytoma cell line P3/NS1/1-Ag4-1. An indirect immunofluorescence assay (described in section E below) was used to detect clones producing MAb which react with the immunizing cell population. Such clones were then tested against a panel of hematopoietic cell populations. MAb J3-119 reacts exclusively with B cells. The hybridoma was then subcloned by limiting dilution without feeder cells and retested. MAb from original and subcloned hybridomas were compared for their ability to bind to the immunizing cell population under conditions of antibody excess to assess the possible presence of multiple antibody-producing clones in the original hybridoma. Such antibodies were considered to be identical if they produced identical immunofluorescence

profiles when assayed in parallel and failed to produce additive binding patterns with the use of an indirect immunofluorescence assay. Serotyping indicates that J3-119 is of the IgG_1 subclass.

B. Antibody Purification

Antibodies were obtained from the ascites of tumor-bearing mice. MAb were purified from murine ascites using a protein A-Sepharose column (Sigma Chemical Co., St. Louis, Mo.). IgG_1, MAb were loaded under high salt, high pH conditions (0.1 M glycine, 3 M NaCl, pH 8.9), and eluted with 0.1 M pH 6.0 citrate buffer. After elution, MAb were exhaustively dialyzed against pH 7.4 PBS. Antibody purity was confirmed by demonstration of single heavy and light chain Ig bands on SDS-PAGE.

C. Antibody Radiolabeling

Affinity purified MAb (250 μgm) was trace-labeled with ^{125}I (500 μCi) using Iodo-Beads (Pierce Chemical Co., Rockford, IL) with removal of free ^{125}I by gel filtration. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Specific activities obtained ranged from 0.95 to 1.04 μCi/μgm. Immunoreactivity was determined by incubating ^{125}I-labeled antibody (10 mg/ml) with increasing numbers of cells to 2.5×10^7/ml.

D. Fluorescein Conjugation

Twenty μl of fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, Oreg.) in 1 M $NaHCO_3$ (3 mg/ml) at pH 9.5 was added to 200 μl of affinity purified MAb (10 mg/ml) in PBS and incubated for two hours at room temperature on a mechanical rotator. Free FITC was removed by filtration on a Sephadex G-25 column. Molar fluorescein:protein ratios were in the 6-10 range. FITC-labeled MAb were screened on antigen positive and negative cell lines by flow cytometry to determine optimum working dilutions.

E. Immunofluorescence Analysis

Antibody binding to target cells was determined by using either directly fluoresceinated MAb or by employing fluoresceinated goat anti-mouse antibody (Tago, Burlingame, Calif.) in a standard indirect immunofluorescence binding assay. Antibody binding in both assays was monitored on the Epics Profile cytofluorograph (Coulter, Hialeah, Fla.) or the Fluorescence Activated

Cell Sorter (FACS) (Becton-Dickinson, Mountainview, Calif.) with fluorescence displayed in the logarithmic mode. All procedures were performed in the presence of 0.1% NaN_3 to prevent antigenic modulation. Background fluorescence for indirect immunofluorescence assays was determined by incubating cells with nonreactive isotype-matched MAb.

F. Antigen Radiolabeling

Surface glycoproteins were labeled with ^{125}I using the lactoperoxidase method or with NaB^3H_4 using the nueraminidase/galactose oxidase method (e.g., Gahmberg and Hakomori, J. Biol. Chem. 250:2447-51, 1975). Cells (2×10^8) were treated with neuraminidase (Sigma Chem. Co., St. Louis, Mo., 0.1 U/ml) and galactose oxidase (Sigma, 80 U total) at 37°C for one hour, washed, and incubated with 50 mCi of NaB^3H_4 for 30 minutes at room temperature. After washing, cells were lysed (0.05 M Tris, 0.14 M NaCl, 1mM $MgCl_2$. One percent Triton X-100, 1 mM PMSF, pH 8.0) for 30 minutes on ice. Nuclei were pelleted at 15,000 x g and lysates were stored at -80°C until use.

Cells to be metabolically labeled with $^{32}PO_4$ were washed and incubated in phosphate-free MEM medium (GIBCO) for 2 hours at 37°C. They were then resuspended in phosphate-free medium at 1×10^7/ml and incubated with 1-2 mCi of $^{32}PO_4$ (Amersham, Arlington Heights, Ill.) for 1 hour at 37°C. After washing, cells were lysed as described above, except that 50 mM NaF, 5 mM sodium pyrophosphate, and 2 mM sodium orthovanadate were added to inhibit phosphatase enzymes. Lysates were passed through a Sephadex G-25 column (Pharmacia, Piscataway, N.J.) to remove free phosphate.

G. Immune Precipitation

Radiolabeled detergent solubilized cell lysates were applied to a Sephadex G-25 column to isolate radiolabeled proteins. Lysates were then precleared by incubating them with affinity purified rabbit anti-mouse Ig that had been covalently coupled to Sepharose CL4B (R/M-Sepharose). Individual MAb were incubated overnight in parallel at 4°C with R/M-Sepharose. Antibody-matrix complexes were then washed and incubated for 16 hours at 4°C with aliquots of the appropriate precleared radiolabeled lysate. Samples were then extensively washed (0.05 M Tris-HCl, 0.1% Triton X-100, 140 mM NaCl, pH 8.0) and antigens eluted by heating at 100°C for 3 minutes in reducing sample buffer. Antigens were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10%-15% gradient slab gel and visualized by

autoradiography. Molecular weight standards labeled with ^{14}C-acetate were purchased from Amersham (Arlington Heights, IL) and included myosin (200,000), phosphorylase B (97,000), albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

SKLY18 cells metabolically labeled with $^{32}PO_4$ were again incubated in parallel with either an isotype-matched control MAb or anti-gamma (50 μgm/ml) under modulating conditions. Equal numbers of cells were harvested and $^{32}PO_4$-labeled CD19 antigens isolated and compared by immune precipitation at 0, 30, or 90 minutes. Autoradiographs of SDS gels of the immune precipitations show that CD19 is intensely labeled with ^{32}P and, therefore, indicate that this transmembrane protein is phosphorylated. Weak but consistent and specific ^{32}P-labelling of CD45 is also noted under these conditions, indicating that it too is a phosphoprotein. Comparative studies indicate that there is approximately 70% as much ^{32}P-labeled CD19 isolated from anti-Ig-treated cells versus anti-CD4-treated cells at both 60 and 90 minutes.

EXAMPLE 2

Rapid and Specific Internalization of Anti-CD19 MAb

A. <u>Antigenic Modulation</u>

The SKLY18 B lymphoma cell line was cultured in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hyclone), glutamine (GIBCO), nonessential amino acids (GIBCO), and penicillin/streptomycin (GIBCO) at 37°C in a 5% CO_2 atmosphere with 100% humidity. Viability of all cells used in these experiments was greater than 95%.

Modulation of surface antigens was performed by incubating cells (1 x 10^6/ml) under standard tissue culture conditions with 10 μg/ml of purified MAb for the times indicated. Identical cultures were incubated with isotype-matched MAb to sIg, CD19, or CD4 antigens at 37°C, washed, incubated with MAb specific for test antigens at 4°C, and analyzed. Changes in the binding of directly fluoresceinated MAb are expressed by dividing the mean channel of fluorescence observed on reactive-antibody treated cells by that observed with control (anti-CD4) treated cells after correcting for nonspecific binding.

For indirect immunofluorescence assays, murine antibodies remaining on the cell surface after the 37°C incubation were detected by using fluoresceinated goat anti-mouse antibody (G/M-FITC) at 4°C. All modulated cell populations were also incubated with additional test MAb at 4°C followed by

G/M-FITC to detect free antigen remaining on the cell surface. Percent modulation is calculated by subtracting the corrected mean channel of fluorescence observed with the test MAb for cells in the antibody-treated culture from that of cells in the control culture and dividing by the latter.

As shown in Figure 4, coincubation of cells with both anti-Ig and anti-CD19 MAb selectively increases the rate and extent of CD19 modulation. CD19 expression is monitored on SKLY18 cells incubated with the following MAb (each at 10 μgm/ml) at 37°C: anti-CD4 (J5-19), anti-Ig (HB43), anti-CD19 (J3-119), and anti-Ig plus anti-CD19. The percent of detectable CD19 remaining on the cell surface at 2.25 and 5 hours is indicated. Incubating cells with MAb to either sIg or CD19 reduces expression of CD19; incubating cells with MAb to both antigens simultaneously increases both the rate and extent of CD19 modulation. CD19 expression is followed using an indirect immunofluorescence assay and confirmed using directly fluoresceinated anti-CD19 MAb when anti-CD4 and anti-IgG MAb are the modulating agents. Modulation of sIgG on SKLY18 by anti-IgG MAb is greater than 95% complete by 2 hours, permitting accurate assessment of CD19 expression using directly fluoresceinated reagents.

B. Capping Studies

Cells are preincubated with directly fluoresceinated MAb to test antigens at 4°C for 20 minutes, washed, and then coincubated with either unlabeled anti-lambda or isotype-matched anti-CD4 MAb (30 μgm/ml) at 37°C. At selected time intervals, aliquots of cells were removed, washed at 4°C, treated with sodium azide, and analyzed using a laser scanning confocal microscope (MRC-500; Bio-Rad Laboratories, Cambridge, Mass.) with a Nikon Optiphot (Nikon, Torrance, Calif.). Field depths are 1 μm.

Cocapping studies (as shown in Figure 3) confirm that anti-CD19 MAb is rapidly capped, internalized, and degraded when cells are coincubated with anti-Ig MAb. Intensity of fluoresceinated caps decreases after internalization as cells become diffusely and then less fluorescent. In contrast, cells incubated with fluoresceinated anti-CD19 MAb and unlabeled anti-CD4 control MAb show minimal cap formation or internalization of fluorescein label. Similarly, distribution of fluoresceinated anti-CD45 control MAb is identical in cells coincubated with anti-CD4 or anti-Ig MAb. Cells are pretreated with fluoresceinated anti-CD19 MAb at 4°C, washed, and coincubated with either isotype-matched anti-CD4 MAb (Panels A-D) or anti-lambda MAb (Panels E-H) at 37°C. Cells were removed, washed, and analyzed at 0, 20, 40, 60, 90, and 120

minutes. Results at 40, 60, 90, and 120 minutes are indicated in panels A and E, B and F, C and G, and D and H, respectively. In cells treated with anti-lambda MAb, patches are visible by 20 and 40 minutes, internalization of caps is clearly seen by 60 minutes, cells have become diffusely fluorescent by 90 minutes, and by 120 minutes intracellular aggregates are less distinct and cells are paler, reflecting degradation of the labeled MAb. In contrast, cells coincubated with anti-CD4 MAb show minimal patch formation or internalization of fluoresceinated MAb.

C. Internalization of Radiolabeled Anti-CD19 MAb

^{125}I-labeled J3-119 anti-CD19 MAb (10 μgm) was added to 60 x 10^6 SKLY18 cells and incubated for 40 minutes at 4°C. Cells were then washed three times in cold media and resuspended at 1 x 10^7 cells/ml. 1 x 10^6 cells (100 μl) were added to a series of tubes containing 400 μl of RPMI 1640 media with 0.1% bovine serum albumin and either anti-lambda or anti-CD4 MAb (both at 15 μgm/ml) and incubated at 37°C for varying times. At each time point, 3 tubes (3 x 10^6 cells) from both the anti-lambda and control lots were removed, centrifuged at 4°C, and washed once, saving both supernatants for counting. One of the tubes was used to determine both trichloroacetic acid (TCA) precipitable and TCA nonprecipitable counts released into the media. The other two tubes were incubated with pH 2.8 RPMI 1460 media for ten minutes on ice. Cells were then pelleted and washed again with pH 2.8 RPMI 1640 media. Supernatants from these two acid washes were pooled and counted. The cell pellets from these acid washes were also saved and counted.

As shown in Figure 1, radiolabeled anti-CD19 MAb is rapidly removed from the cell surface, internalized, and then degraded, with release of antibody fragments into the tissue culture media, in the presence of anti-Ig but not anti-CD4 MAb. Identical cell cultures are incubated in parallel with ^{125}I-labeled anti-CD19 MAb (J3-119). Cells are also incubated with 15 μgm/ml of either anti-CD4 control MAb (Figure 1A) or anti-Ig MAb (Figure 1B). All other conditions are the same. Aliquots from the two cultures are removed at the times indicated and assayed for radiolabeled internalized, cell-bound, and free MAb (intact and antibody fragments). Incubation of cells with ^{125}I-labeled anti-CD19 MAb plus anti-CD4 MAb leaves most anti-CD19 MAb on the cell surface (Figure 1A). Little MAb enters cells or becomes fragmented. In contrast, coincubation with unlabeled anti-Ig MAb causes rapid internalization and degradation of iodinated anti-CD19 MAb (Figure 1B). Loss of surface anti-CD19 MAb is accompanied by

a rapid rise and subsequent slow decrease in internalized MAb, with a maximum achieved at 15-30 minutes.

The results of Figure 2 confirm that B cells incubated with anti-Ig MAb at 37°C rapidly internalize and degrade this antibody. SKLY18 lymphoma cells are incubated with ^{125}I-labeled anti-Ig MAb at 37°C as described in Figure 1. Aliquots are removed at the times indicated and assayed for internalized, cell-bound, and free MAb (intact and antibody fragments).

Tables I and II quantify the data in Figures 1 and 2. The percent of degraded, radiolabeled MAb (Table I) reflects the amount of MAb that has passed through the cell during the course of the experiment.

TABLE I

Percent of Internalized Antibody

MAb/Time	0	15	30	45	60	80	100
αIgG	8.0	74.9	70.9	59.5	45.9	35.5	25.7
αCD19/αIgG	10.8	38.8	40.7	29.8	23.3	13.8	11.8
αCD19/αCD4	10.8	6.7	6.9	6.9	7.4	9.6	9.1

The amount of cell-associated and acid wash-resistant radioactivity in Figures 1 and 2 is expressed as a percent of the total amount of recovered radioactivity at each time point (minutes). Bold characters indicate ^{125}I-labeled MAb.

TABLE II

Percent of Degraded Antibody

MAb/Time	0	15	30	45	60	80	100
αIgG	0.3	2.5	6.5	16.9	31.1	41.9	52.6
αCD19/αIgG	0	2.7	7.5	18.6	30.2	39.4	46.0
αCD19/αCD4	0	<1	1.6	2.6	3.3	4.6	6.3

The amount of TCA nonprecipitable radioactivity in the culture supernatant in Figures 1 and 2 is expressed as a percent of the total amount of recovered radioactivity at that time point (minutes). Bold characters indicate ^{125}I-labeled MAb.

Studies were also performed, using other B cell-reactive MAbs which rapidly modulate expression of their target antigens, to evaluate the specificity of the sIg-CD19 comodulation process. Previous studies of twenty-two B cell surface antigens indicated that most antigens do not undergo significant antibody-induced antigenic modulation. Of those antigens that do modulate, sIg and Tfr do so most efficiently, followed by CALLA/CD10 and then CD10. As shown in Table III, under conditions where CD19 is comodulated by anti-Ig MAb, CD19 is unaffected by specific MAb-mediated modulation of Tfr/CD71 or CALLA/CD10. Therefore, comodulation of CD19 is specifically associated with modulation of sIg by anti-Ig MAb and not with the modulation process itself. On SKLY 18 cells, quantitative expression of sIg and Tfr is compatible while that of CALLA/CD10 is lower.

TABLE III

Specificity of Modulating MAb

Target Antigen	sIg(λ)	Tfr/CD71	CALLA/CD10
CD19 (12.08)	57%	96%	105%
SIg (34.76)	8%	101%	107%
Tfr/CD71 (7.29)	92%	8%	113%
CALLA/CD10 (2.74)	95%	98%	50%
CD45 (16.92)	113%	99%	104%

SKLY18 B lymphoma cells were incubated for 20 hours with purified modulating MAb, washed, and incubated with purified directly fluoresceinated MAb specific for test antigen. Fluorescence was monitored using an Epics Profile cytofluorograph with fluorescence displayed in logarithmic mode. Extent of self-modulation is measured using an indirect immunofluoresce binding assay. Percent change in antigen expression relative to control is shown for a representative experiment. Experiments were performed using isotype-matched modulating MAb. Numbers in parenthesis after each test antigen indicate its mean channel of staining in the control sample as measured using directly fluoresceinated MAb.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

1. The cell line, as designated by ATCC No. HB 10383, that produces monoclonal antibody J3-119.

2. A monoclonal antibody produced by the cell line of claim 1.

3. A monoclonal antibody that competitively inhibits the formation of an immunocomplex between the antibody of claim 2 and the CD19 antigen.

4. An immunoconjugate comprising at least one agent coupled to a monoclonal antibody produced by the cell line of claim 1.

5. An immunoconjugate comprising at least one agent coupled to a monoclonal antibody according to claim 3.

6. An immunoconjugate according to either one of claims 4 and 5 wherein the agent is a diagnostic agent or a therapeutic agent.

7. An immunoconjugate according to either one of claims 4 and 5 for use as an active therapeutic substance.

8. An immunoconjugate according to either one of claims 4 and 5, in combination with an antibody that selectively binds to the surface immunoglobulin of a target B cell, for use as an active therapeutic substance.

9. An immunoconjugate comprising at least one diagnostic agent coupled to an antibody that selectively binds to the CD19 antigen, in combination with an antibody that selectively binds to the surface immunoglobulin of a target B cell, for use within a method for detecting a B cell associated with a B cell disorder.

10. An immunoconjugate comprising at least one therapeutic agent coupled to an antibody that selectively binds to the CD19 antigen, in combination with an antibody that selectively binds to the surface immunoglobulin of a target B cell, for use within a method for treating a B cell disorder in a warm-blooded animal.

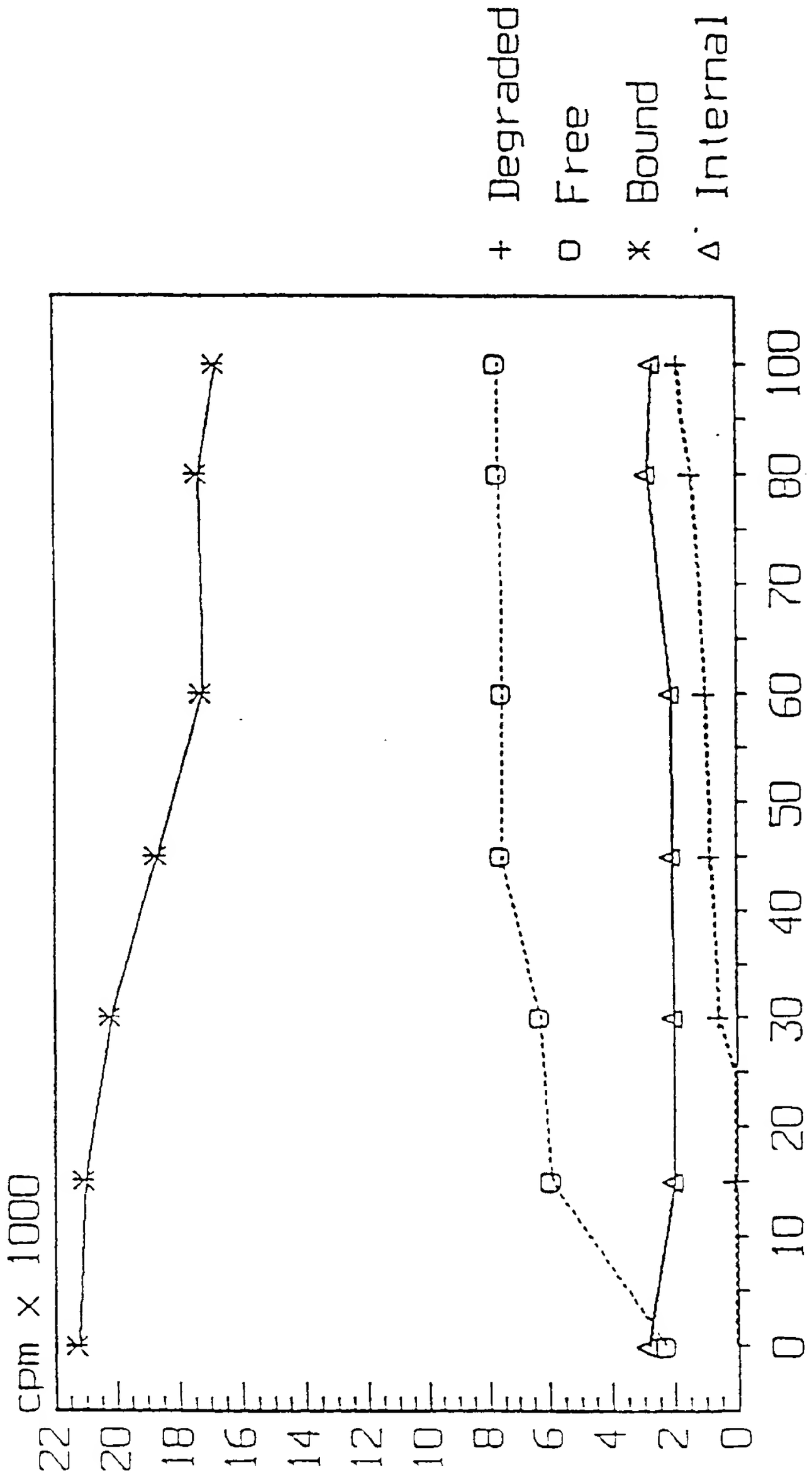
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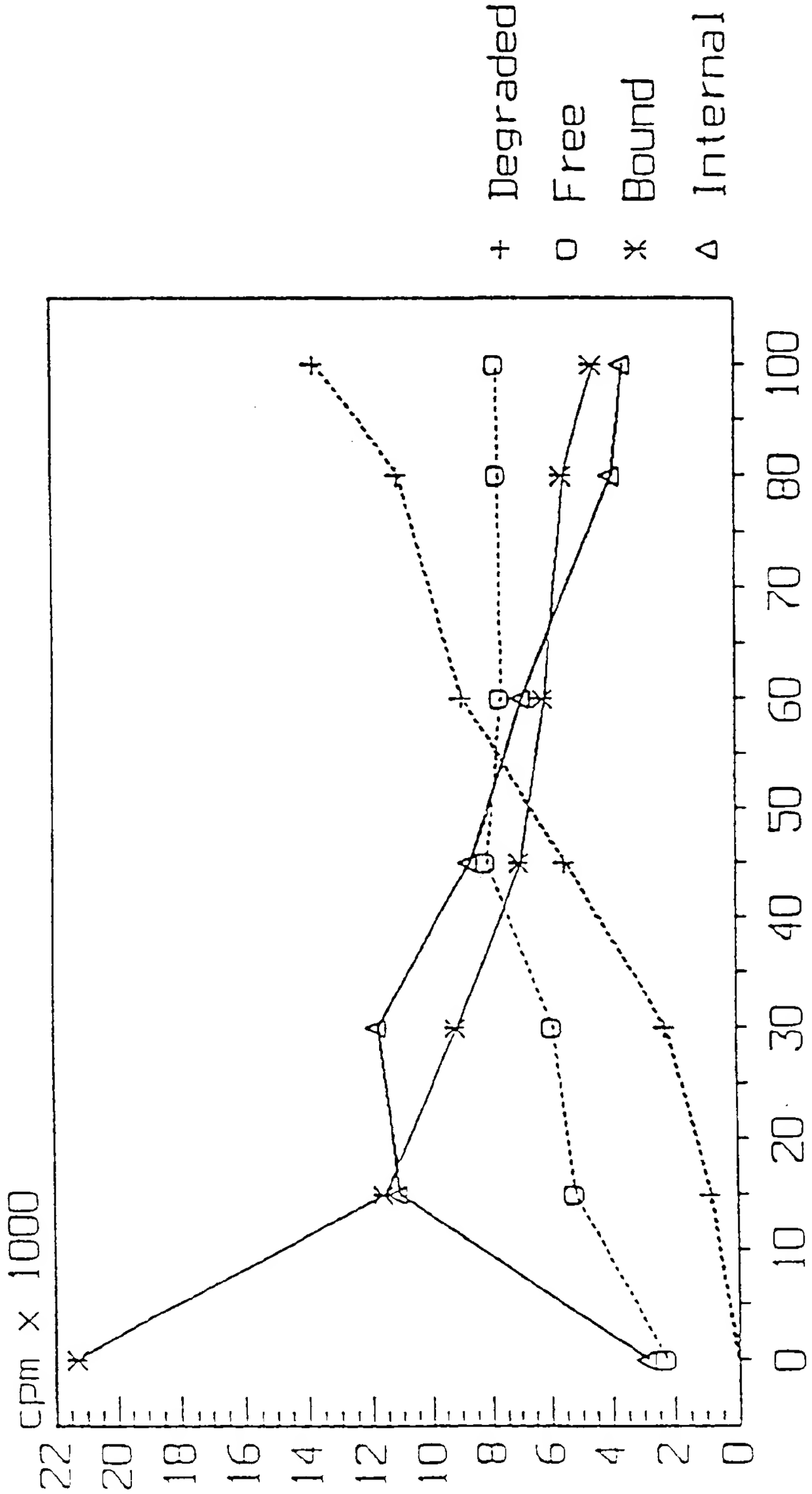
FIGURE 1A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Time (min)

FIGURE 1B

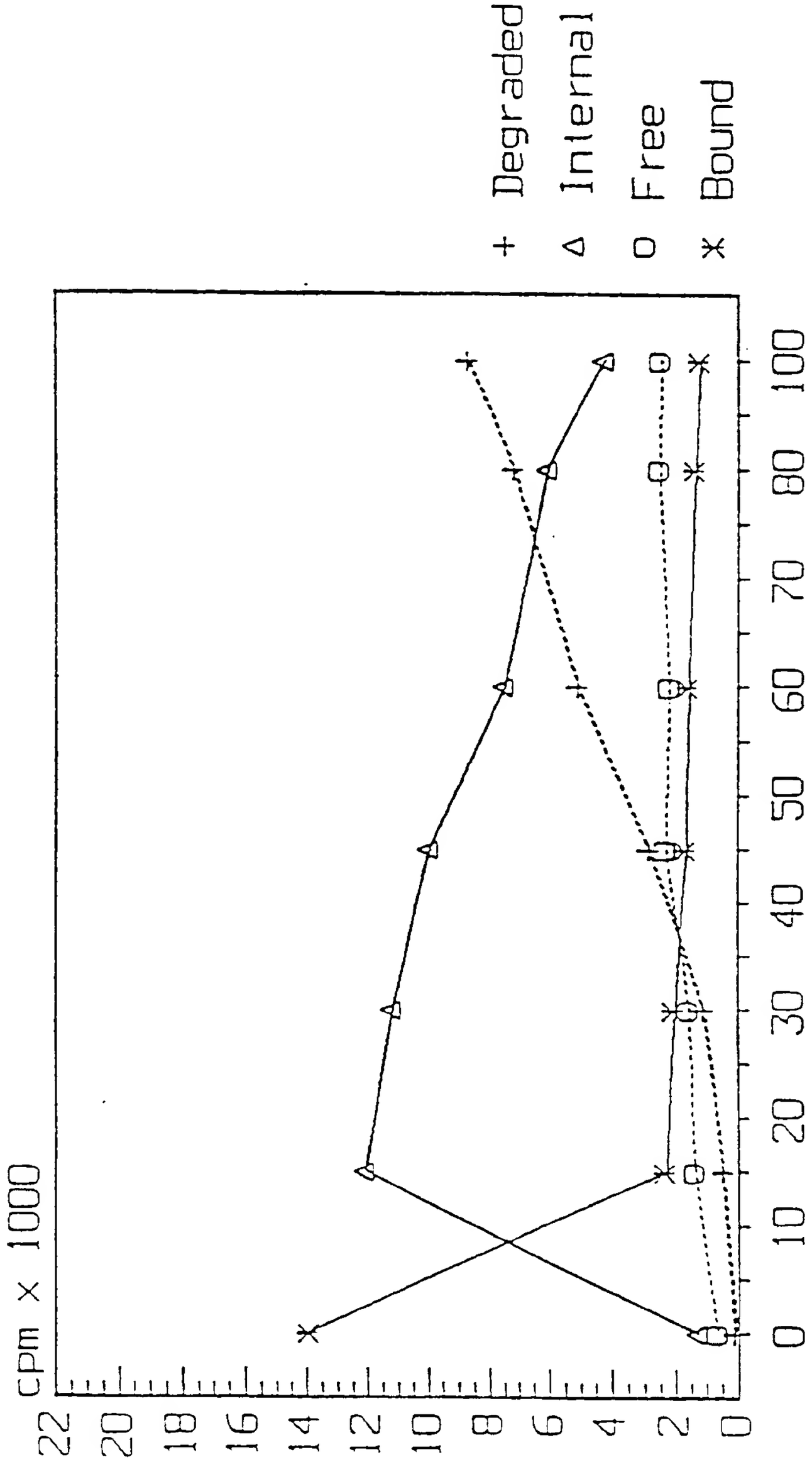
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FIGURE 2

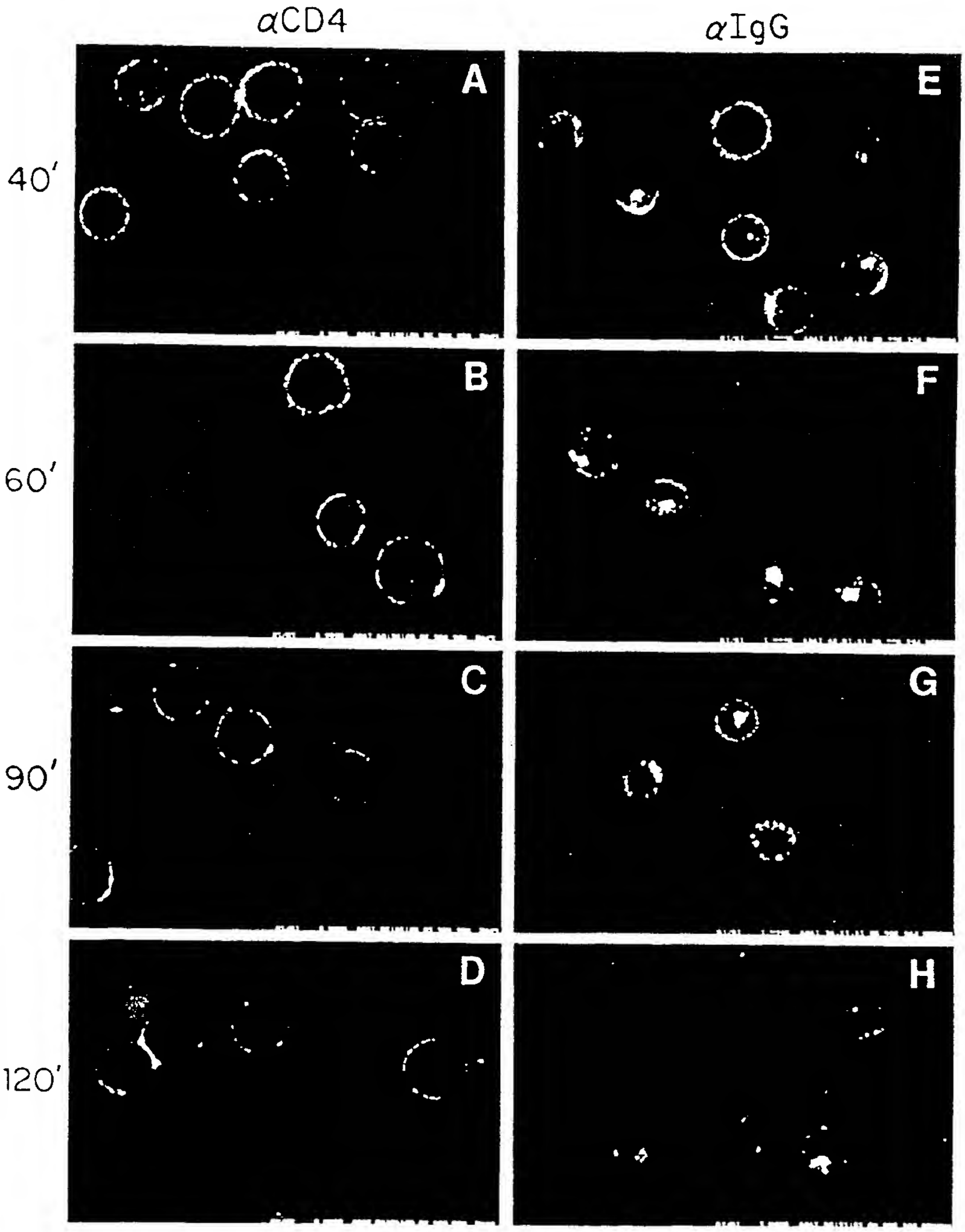
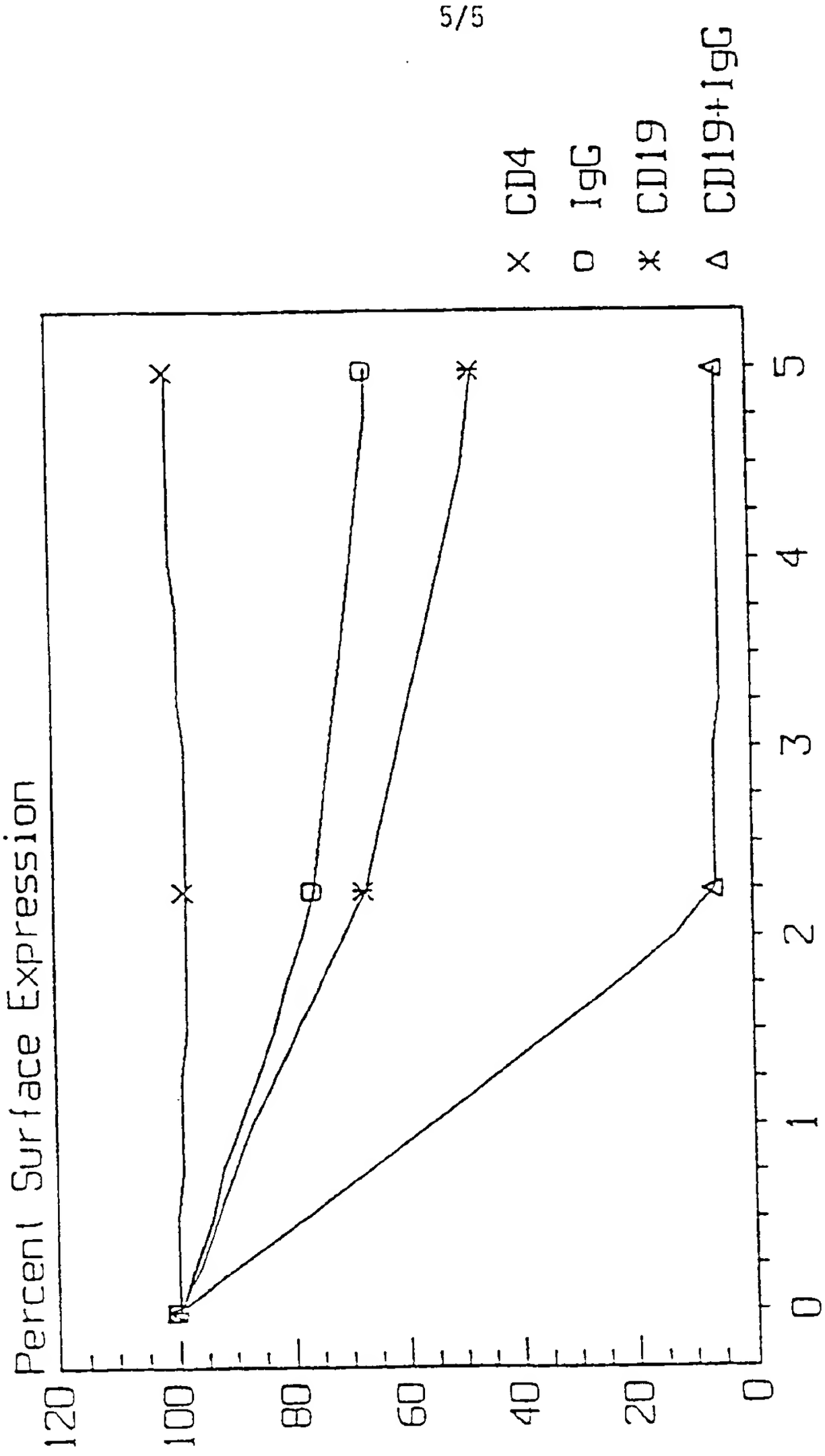


FIG. 3


X CD4
O IgG
* CD19
Δ CD19+IgG
Percent Surface Expression
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100
80
60
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1
2
3
4
5
Time (hrs)

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/01649

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N5/20 ; C12P21/08 ; A61K39/395 ; A61K39/44

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	THE JOURNAL OF EXPERIMENTAL MEDICINE vol. 170, no. 6, 1 December 89, pages 2159 - 2164; J.M.PESANDO: "CD19 is functionally and physically associated with surface immunoglobulin" see page 2160:"results" ---	1-10
X	TISSUE ANTIGENS vol. 33, no. 2, 1989, page 136 J.M.PESANDO: "Comparison of the epitope specificity of 14 anti-CD19 monoclonal antibodies" see page 136:"abstract" ---	2, 3
X	EP,A,317156 (BECTON DICKINSON) 24 May 1989 see column 6, line 11 - column 8, line 17	4-7
Y	---	8-10
	-/--	

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01 JULY 1991	08 08. 91
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	TURMO Y BLANCO

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with Indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	US,A,4661586 (LEVY) 28 April 1987 see column 1, line 58 - column 2, line 45 ---	8-10

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 91/01649

SA 45957

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-317156	24-05-89	JP-A- 1161153	23-06-89
US-A-4661586	28-04-87	US-A- 4816249	28-03-89